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ORIGINAL PAPER

Direct Analysis of Per- and Polyfluoroalkyl Substances (PFAS) in Plasma by Liquid Chromatography Coupled to Mass Spectrometry (LC–MS) for Biomonitoring Purposes

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Abstract

A rapid and direct liquid chromatography–mass spectrometry (LC–MS) method was developed and validated for high-throughput biomonitoring of Per- and Polyfluoroalkyl Substances (PFAS) in human plasma. This approach streamlines the analytical workflow by replacing traditional, labor-intensive solid-phase extraction (SPE) with a simple protein-precipitation step. Twenty-three PFAS (PFBA, PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnA, PFDoA, HFPO-Da, PFMPA, PFMBA, PFBS, PFPeS, PFHpS, PFOS, ADONA, 4:2 FTS, 6:2 FTS, 8:2 FTS, 9Cl-PF3ONS, 11Cl-PF3OUdS, PFEESA) were monitored. Method detection limits (MDLs) and limits of quantification (LOQs) ranged from 0.01 to 0.39 ng/mL and from 0.04 to 1.2 ng/mL, respectively. Accuracy (bias) and precision (RSD) were below 16% and 10%, respectively, for all PFAS in both intra- and inter-day experiments. Recoveries ranged from 86 to 102%, and matrix effects were estimated as ion suppression below 30% for all analytes. The method was successfully applied to a cohort of 426 Italian citizens who participated on a voluntary basis. Twelve PFAS were detected, with PFOS and PFOA present in 95% of the samples. The mean and median sums of detected PFAS were 7.3 ng/mL (SD 4.6) and 6.2 ng/mL $IQR 4.4-9.2$, respectively, with a maximum total concentration of 35 ng/mL. PFOS isomers (linear+branched) contributed the most to the total PFAS burden in plasma, reaching a maximum concentration of 25 ng/mL. Overall, this method proved to be a reliable and efficient tool for large-scale biomonitoring studies and provides an important basis for investigating the long-term health implications of PFAS exposure.

Keywords PFAS · Per- and Polyfluoroalkyl substances · LC–MS · Direct analysis · Biomonitoring

Introduction

Per- and polyfluoroalkyl substances (PFAS) are a growing concern within environmental and public health spheres due to their persistent nature and potential adverse health effects. These synthetic compounds, used for a variety of industrial applications due to their water- and oil-repellent properties, pose significant biotoxicological challenges. The increasing prevalence of PFAS in the environment, especially in water supplies (Domingo et al., 2019) and consequently in human specimens (Piva et al. 2021; Pascali et al. 2023; Cao and Ng 2021; Ríos-Bonilla et al. 2024; Fenton et al. 2021) highlights the urgent need for accurate biomonitoring techniques. Biomonitoring studies aim at measuring chemicals, their metabolites or biomarkers in human tissues, fluids, or exhaled breath to assess environmental exposures. Urine and blood are the specimen of choice in toxicology studies, able to reflect exposure to chemicals within the past 24–48 h, but while blood samples require more invasive procedures, urine is advantaged in biomonitoring studies due to the ease of collection. Unfortunately, some contaminants, such as PFAS, are not properly monitored in this matrix because they are difficult to eliminate through the renal pathway

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and blood or blood derivates remain the matrix of choice (Yu et al., 2020). Regardless of the matrix, biomonitoring studies are a powerful tool in environmental health science, serving several critical functions. They are used to i) establish reference values from a general population; ii) identify elevated exposure compared to reference values; iii) support epidemiological research, helping to investigate potential links between chemical exposures and diseases and iv) to assess the effectiveness of public health interventions in reducing hazardous chemical exposures. The analysis of PFAS in human blood and/or blood derivatives is challenging due to the compounds' high affinity for serum albumin and other proteins. In this view, to address cleanliness, solid phase-extraction (SPE) had become the preferred method in high-sensitivity workflows. Weak Anion Exchange (WAX) sorbents were considered the "gold standard" for blood matrices; they combined ionic and hydrophobic interactions allowing for selective retention of both acidic and neutral compounds. Under these conditions, the obtained recovery for most of compounds were in the range 65–84% and sensitivity was below 0.5 ng/ml (Kaiser et al. 2021), strongly dependent on chemical structure of the molecule. Different performances have been reported for sensitivity calculated in plasma and serum samples, with slightly higher sensitivity in plasma (Ao et al., 2023). In addition to WAX-based sorbents, SPE based on hydrophilic-lipophilic balance (HLB) interaction has been successfully implemented, achieving recovery rates ranging from 30 to 91% with sensitivity between 0.2 and 3.2 ng/ml. A primary advantage of the SPE-HLB approach is its versatility; the same standardized protocol can be efficiently adapted to extract PFAS from diverse biological matrices (such as plasma and milk) with minimal modifications. This cross-matrix compatibility not only streamlines laboratory workload but also ensures high comparability across results from different types of samples (Kuklenyik et al. 2004). HLB sorbents have been exploited also for the development of microextraction methodologies in order to support sustainability, reaching sensitivity below 1 ng/ml for 18 PFAS (Li et al. 2026). In terms of reducing matrix effects and maintaining instrument performance over time, SPE is regarded by many researchers as superior to other preparation techniques due to its superior cleanup efficiency. Recent literature highlights a shift toward online SPE coupled to LC–MS. Unlike traditional offline SPE, which is manual and time-consuming, online systems automate the clean-up, significantly reducing sample volume (down to 25–100 µL) and contamination risks (Workman et al. 2019; Belay et al., 2025). However, to be statistically significative, biomonitoring studies should involve huge number of samples and for this reason the applied approaches should be cost effective, rapid, easy to perform and robust. Analytical methods such as liquid chromatography- mass spectrometry (LC–MS) and the commercially available introduction of very sensitive instrumentation have facilitated massive progress in detecting these substances at very low level limiting extensive sample preparation such as solid-phase extraction (EPA 537; Szabo et al. 2022; Piva et al. 2023) and favoring faster approaches such as liquid–liquid extraction.

In this frame, a method based on direct injection of plasma after solvent protein precipitation was developed for the determination of 23 PFAS, among legacy and emerging congeners. Quantification exploited the isotope dilution approach, considered the gold standard in quantitative mass spectrometry. This approach involved spiking the sample with a known amount of PFAS isotopically labeled internal standards prior to precipitation, allowing for accurate compensation of any analyte loss or variability in ionization efficiency caused by the complex plasma matrix. To prove the effectiveness of the method, it has been applied to 426 real samples collected voluntarily from Italian citizen undergone biomonitoring voluntarily. The study's results were contextualized within the National Academies of Sciences, Engineering, and Medicine (NASEM) guidelines (NASEM, 2022), which provided a tiered framework for interpreting PFAS blood concentrations in a clinical setting.

Materials and Methods

Materials

Analytes and isotopically labelled standards (> 98 purity) were purchased from Wellington Laboratories (Guelph, Canada). Native analytes included perfluoro-*n*-butanoic acid (PFBA); perfluoro-*n*-pentanoic acid (PFPeA); perfluoro-*n*-hexanoic acid (PFHxA); perfluoro-*n*-heptanoic (PFHpA); perfluoro-*n*-octanoic acid (PFOA); perfluoro-*n*-nonanoic acid (PFNA); perfluoro-*n*-decanoic acid (PFDA); perfluoro-*n*-undecanoic acid (PFUnA); perfluoro-*n*-dodecanoic acid (PFDoA); tetrafluoro-2-heptafluoropropoxy propanoic acid (HFPO-Da); perfluoro-3-methoxypropanoic acid (PFMPA); perfluoro-4-methoxybutanoic acid (PFMBA); potassium-perfluoro-1-butanesulfonate (PFBS); sodium perfluoro-1-pentanesulfonate (PFPeS); potassium perfluorohexane sulfonate(PFHxS); sodium perfluoro-1-heptanesulfonate (PFHpS); potassium perfluoro-1-octanesulfonate (PFOS); sodium dodecafluoro-3H-4,8-Dioxanonanoate (DONA); 1H,1H, 2H,2H-perfluoro-1-hexanesufonate (4:2 FTS); 1H,1H, 2H,2H-perfluoro-1-octanesufonate (6:2 FTS); 1H,1H, 2H,2H-perfluoro-1-decanesufonate (8:2 FTS); potassium 9-chlorohexadecafluoro-3-oxanonane-1-sulfonate (9Cl-PF3ONS); potassium 11-chloroeicosafluoro-

3-oxaundecane-1-sulfonate (11Cl-PF3OUdS); potassium perfluoro (2-ethoxyethane) sulfonate (PFEESA).

Isotopically labelled internal standards (surrogates) were added to the sample before extraction in a known amount to compensate for analyte losses and matrix effects during the extraction process. Since not all target PFAS have an isotopically labelled analogue, an alternate labelled compound is used as recommended in EPA-533 method (see Table 1 in SI).

Isotopically labelled standards were PFBA $^{13}C_4$ (MPFBA) used to quantify PFBA and PFMPA; PFPeA $^{13}C_5$ (M5PFPeA) used to quantify PFPeA and PFMBA; PFHxA $^{13}C_5$ (M5PFHxA) used to quantify PFHxA; PFHpA $^{13}C_4$ (M4PFHpA) used to quantify PFHpA and ADONA; PFOA $^{13}C_8$ (M8PFOA) used to quantify PFOA; PFNA $^{13}C_9$ (M9PFNA) used to quantify PFNA; $^{13}C_6$PFDA (M6PFDA) used to quantify PFDA, PFUnA $^{13}C_7$ (M7PFUnA) used to quantify PFUnA; PFDoA $^{13}C_2$ (MPFDoA) used to quantify PFDoA; HFPO-DA $^{13}C_3$ (M3HFPO-DA) used to quantify HFPO-DA, PFBS $^{13}C_3$ (M3PFBS) used to quantify PFBS and PFEESA; PFHxS $^{13}C_3$ (M3PFHxS) used to quantify PFHxS and PFPeS; PFOS $^{13}C_8$ (M8PFOS) used to quantify PFOS, 9Cl-PF3ONS, 11Cl-PF3OUdS and PFHpS; 4:2FTS $13C_2$ (M2-4:2FTS) used to quantify 4:2 FTS; 6:2 FTS $13C_2$ (M2-6:2FTS) used to quantify 6:2 FTS and 8:2 FTS $13C_2$ (M2-8:2FTS) used to quantify 8:2 FTS.

Mass-labelled perfluoro-n- [2,3,4 $^{13}C_3$] butanoic acid (M3PFBA); perfluoro-n-[1,2-$^{13}C_2$] octanoic acid (M2PFOA), and mass-labelled sodium perfluoro-1-[1,2,3,4-$^{13}C_4$] octanesulfonate (MPFOS) at chemical purities > 98% and isotopic purities of ≥ 99% were used as injection standards. They were added to the purified extracts before injection to monitor instrument stability and provide a reference for the precision of the injection.

Target perfluoroalkyl analytes, surrogate mass-labelled compounds and injection standards were at the following concentration: 500 ng/ml, 500-2000 ng/ml, 1000-3000 ng/ml. Working solution of surrogate mass-labelled compounds and injection standards solutions were prepared at the concentration of 25–100 ng/ml and 100–300 ng/ml, respectively, in methanol and stored at -20 °C.

Methanol, acetonitrile and formic acid 98–100% (all LC–MS grade) were supplied by Merck (Darmstadt, Germany). Ultrapure water (18.2 MΩ·cm resistivity) for mobile phase and sample preparation was produced by Sartorius Arium mini apparatus (Sartorius, Goettingen, Germany). Ammonium acetate was provided by Sigma-Aldrich (S.Louis, MO, USA). Human blank serum to experimentally check LOQ and MDL was acquired by Merck (Darmstadt, Germany).

Sample Collection

Plasma samples for validation testing were obtained from non-fasting volunteers (n. 3) by collecting whole blood in EDTA containing tubes after venipuncture from the antecubital vein. Plasmas were screened for PFAS concentration values, especially PFOA and PFOS, which are the most frequently detected compounds at higher concentration in the general population. Two of the volunteers exhibited acceptable concentration of PFOA and PFOS and were selected for validation tests (< 1 ng/ml). The robustness and suitability of the method for large-scale biomonitoring were verified through the analysis of 426 plasma samples from participants recruited voluntarily within the general population in central Italian region. The study was approved by the Bioethical Committee of University of Bologna (protocols number 0041730 28/11/2023 and 0007486 01/08/2025). Procedural blank samples were obtained by using water as a test matrix collected into vacutainer tubes and processed as samples. Plasma samples were stored in freezer at -20 °C until analysis.

Sample Preparation

Aliquots of 200µL of plasma samples and quality controls (QC) were spiked with 2,5µL of an isotopically labeled standard working solution (surrogate mass-labelled compounds, final concentration 6.25–25 ng/ml). Protein precipitation was performed by adding 400 µL of 1% formic acid in acetonitrile to each sample, followed by immediate and vigorous vortex-mixing for 30 s. Samples were centrifuged for 10 min at 14,000 rpm. The supernatant was diluted 1:2 (v/v) with ultrapure water (18.2 MΩ·cm, Sartorius Arium mini). Injection standards (2 µL, final concentration 3.3-10 ng/ml)) were added automatically by the autosampler's injection program during the sample's injection into the LC–MS/MS system. QC samples and calibration standards were prepared following the same procedure described for the plasma samples. QC samples were prepared at the concentrations of 2 ng/ml and 3.5 ng/ml for each analyte in plasma. Five different calibration curves were prepared in solvent at concentrations of 0.1, 0.2, 0.5, 1, 2.5, 3, 5, and 10 ng/ml for each analyte.

Instrumentation

Analyses were carried out in a LC–MS/MS system composed of 1290 Infinity II coupled to a 6495-triple quadrupole (Agilent Technologies, Santa Clara, USA) via ESI source.

Chromatographic separations were achieved in a Poroshell EC-C18 column (2.1 × 100 mm, 1.9 µm), (Agilent Technologies) at 50 °C. The mobile phase consisted of

Table 1 Method perfomance parameters are reported in the table below

Analyte	RT	MDL (ng/ml)	LOQ (ng/ml)	Calibration range (ng/mL)	Recovery (%)	Matrix effect (%)	Intraday QC @ 2 ng/ml % bias (cv)	Interday QC @ 2 ng/ml % bias (cv)	Intraday QC @ 3.5 ng/ml % bias (cv)	Interday QC @ 3.5 ng/ml % bias (cv)
11Cl-PF3OUdS	12.30	0.08	0.26	0.30–5.00	–	–	5 (1)	14 (3)	1 (2)	14 (1)
4:2FTS	4.84	0.25	0.83	1.00–5.00	95	-30	8 (1)	10 (5)	3 (0.5)	6 (6)
6:2FTS	7.21	0.17	0.50	0.50–5.00	80	-22	4 (1)	13 (5)	10 (2)	15 (3)
8:2FTS	9.48	0.39	1.20	1.20–5.00	97	-16	13 (0.4)	16 (6)	7 (7)	9 (6)
9Cl-PF3ONS	10.44	0.04	0.12	0.10–5.00	–	–	4 (0.2)	6 (3)	3 (3)	4 (4)
ADONA	6.81	0.25	0.85	1.00–5.00	–	–	4 (0.2)	11 (3)	2 (0.3)	4 (4)
HFPO-DA	5.62	0.25	0.85	1.00–5.00	101	-25	9 (8)	13 (9)	7 (9)	6 (7)
PFBA	3.16	0.07	0.22	0.25–5.00	92	-28	5 (0.5)	8 (7)	0.3 (0.4)	7 (5)
PFBS	5.00	0.15	0.50	0.50–5.00	95	-23	2 (0.4)	8 (4)	4 (0.4)	9 (4)
PFDA	9.85	0.15	0.51	0.50–5.00	89	-18	3 (4)	13 (2)	2 (0.3)	8 (5)
PFDoA	11.83	0.22	0.74	1.00–5.00	87	-10	2 (0.3)	10 (4)	3 (1)	2 (3)
PFEESA	5.58	0.02	0.06	0.10–5.00	–	–	3 (0.4)	4 (5)	2 80.3)	4 (5)
PFHpA	6.43	0.04	0.12	0.10–5.00	93	-25	4 (0.3)	8 (2)	0.2 (0.4)	3 (3)
PFHpS	8.62	0.12	0.38	0.50–5.00	–	–	3 (3)	9 (2)	2 (4)	4 (3)
PFHxA	5.20	0.11	0.36	0.50–5.00	91	-22	4 (0.1)	3 (4)	5 (0.3)	2 (5)
PFHxS	7.47	0.13	0.43	0.50–5.00	93	-19	4 (7)	9 (6)	2 (1)	8 (7)
PFMBA	4.41	0.06	0.18	0.20–5.00	–	–	5 (0.2)	8 (2)	4 (0.2)	9 (2)
PFMPA	3.49	0.03	0.09	0.10–5.00	–	–	3 (0.1)	7 (3)	4 (0.2)	13 (3)
PFNA	8.76	0.01	0.04	0.05–5.00	87	-22	4 (3)	8 (2)	4 (6)	3 (3)
PFOA	7.62	0.05	0.16	0.20–5.00	90	-30	8 (4)	14 (3)	6 (4)	5 (3)
PFOS	9.71	0.06	0.20	0.20–5.00	102	-22	0.5 (2)	4 (10)	8 (2)	7 (7)
PFPeA	4.08	0.02	0.07	0.10–5.00	86	-24	3 (0.2)	8 (5)	4 (0.2)	13 (3)
PFPeS	6.24	0.06	0.21	0.20–5.00	–	–	1 (0.4)	6 (5)	2 (1)	15 (7)
PFUnA	10.92	0.08	0.26	0.20–5.00	95	-24	9 (1)	14 (3)	2 (0.3)	7 (2)

Table 2 Mean, standard deviation (SD), median and the detection frequency of the retrieved PFAS

Analyte	Mean (ng/ml)	SD	Median (ng/ml)	DF (%)
PFOS	4	3	3.6	95
PFOA	1.20	0.80	1.1	94
PFHxS	0.70	0.60	0.6	70
PFNA	0.30	0.30	0.3	51
PFBA	0.50	1.00	0.7	39
PFHpS	0.71	0.30	0.61	0.8
PFDA	0.34	0.20	0.26	23
PFUnA	0.41	0.20	0.34	9.5
9Cl-PF3ONS	0.16	0.02	0.16	2.1
PFPeA	0.20	-	0.20	0.3
6:2 FTS	0.60	-	0.60	0.3
PFHpA	0.20	0.05	0.17	1.3

10 mM ammonium acetate in water (mobile phase A) and 10 mM ammonium acetate in 75% acetonitrile 25% methanol (mobile phase B). The flow rate was set at 0.3 mL/min. The optimized gradient was as follow: 10% B at time 0 min; 30% B at 1 min, 95% B at 16 min, 10% B at 18 min, 10% B at 21, post run up to 25 min. The injection volume was optimized to 5 µL. Additionally, an InfinityLab PFC Delay Column (4.6 × 30 mm) (Agilent Technologies) was used as a delay column to trap any system related interferences.

Mass spectrometer operated in negative acquisition mode and source parameters were as follow: capillary voltage 3000 V, gas temperature 250 °C, gas flow 11 L/min, nebulizer 25 psi, sheath gas temperature 375 °C, sheath gas flow 11 L/min. Acquisition was performed in multiple reaction monitoring (MRM) by acquiring at least two transitions for each compound, except for PFBA and PFPeA (see Table 1 and 2 in SM for details). Data analysis was carried out by MassHunter software (version 12.0, Agilent Technologies).

Method Validation

The following parameters were tested during validation: selectivity, sensitivity, linearity, accuracy, precision, recovery, matrix effect, dilution integrity and stability of processed samples. Selectivity was tested on 7 different plasma samples checking for interferences from endogenous compounds and/or pharmaceutical drugs at the retention times of the analytes. Quantification of samples was performed by isotope-dilution method and in this view matrix-matched calibration was not required due to the isotope-labelled surrogate used as internal standard for each target analyte. Method linearity was assessed by analyzing five independent calibration curves and processed on five separate days

to evaluate inter-day consistency of relative standard deviation of angular coefficient and intercept (data not shown). The sensitivity of the method, expressed as MDL (method detection limit) was calculated as the minimum concentration of a substance that can be measured and reported with 99% confidence by exploiting the Student's t-Test. The calculation was obtained by analyzing 7 replicates at 2 ng/ml of concentration and evaluating the RSD % of the concentration (EPA 2016, EPA 2018). The limit of quantification (LOQ) was obtained by multiplying the obtained values for 3. LOQ and MDL were finally experimentally verified in spiked human blank serum from Merck. Accuracy and precision data were obtained from QC samples replicates (n.5) on three consecutive days; since PFOA and PFOS were always present in serum, blank subtraction was performed by subtracting the area of the level 0 (no spiking). Recovery was calculated by spiking isotope-labelled surrogate at concentration of 2 and 3.5 ng/ml in plasma. Dilution integrity was evaluated by testing a five-fold dilution on samples prepared at concentrations of 5 and 25 ng/ml, diluted with blank serum and subtracting the contribution of blank for PFOS and PFOA. Procedural blanks were processed in parallel with the samples to evaluate contamination throughout the analytical procedure. Carryover was assessed by injecting a blank sample containing internal standards immediately after a sample with analytes at the upper level of the calibration curve. No carryover was observed for PFAS for the tested concentration above the LOD.

Results

Validation

Analytes eluted within the time range of 3.1 to 12.3 min. For all analytes origin was not included in the calibration curves and 1/x weighting factor was applied. The calibration was linear for the following analytes: PFUnA, PFOS, PFHxS, PFHpS, PFHpA, PFDoA and 9-Cl-PF3ONS; while for PFPeS, PFPeA, PFOA, PFNA, PFMPA, PFMBA, PFHxA, PFEEESA, PFDA, PFBS, PFBA, HFPO-Da, ADONA, 8:2 FTS, 6:2 FTS, 4:2 FTS, 11Cl- PF3OUdS curves were quadratic. All curves had a correlation coefficient R^2 in the range of 0.990–0.999. Method detection limit (MDL) and limit of quantification (LOQ) were in the range 0.01–0.39 ng/ml and 0.04–1.20 ng/ml, respectively. Accuracy bias was calculated using the following equation: $Bias\ (\%) = \left[\frac{(\overline{C} - C_{nom})}{C_{nom}}\right] \times 100$. Precision was expressed as relative standard deviation (RSD %), calculated as: $RSD(\%) = \left(\frac{SD}{\overline{C}}\right) \times 100$. Both parameters were calculated based on the mean of three replicates (n=3) for each concentration level. Accuracy bias and RSD results were below 16% and 10%, respectively for all PFAS both intra and inter-day. Recovery was in the range 86–102%. Matrix-effect was estimated as ion suppression, that occurs when co-eluting matrix components interfere with the ionization process of the analytes leading to a decrease in signal intensity. To evaluate this, we compared the peak areas of analytes spiked into the matrix after extraction with the peak areas of the same analytes in pure solvent: $ME\ (\%) = \left[\left(\frac{Area_{matrix}}{Area_{solvent}}\right) - 1\right] \times 100$. For all analytes, the matrix effect was below 30%. All diluted samples (n. 10) achieved accuracy within 20% respect to the theoretical concentration. Procedural blanks showed no detectable PFAS levels above the Limit of Quantitation (LOQ) (see Table 1 for details). Under the optimized chromatographic conditions, branched isomers of PFOS, PFOA, and PFHxS were adequately resolved from the linear compounds (Fig. 1). For quantification purposes, the peaks of branched and linear isomers were integrated together and reported as total concentration.

PFAS Assessment in Samples

A total of 426 samples were collected for this study. Men accounted for 86% of the recruited samples, age was 1–66 years old. Among the tested PFAS, 12 were detected in our population, namely PFBA, PFPeA, PFHpA, 6:2 FTS, PFHxS, PFOA, PFHpS, PFNA, PFOS, PFDA, PFUnA and 9 Cl-PF3ONS (Table 2). The detection frequency varied from less than 1% for PFPeA, 6:2 FTS and PFHpS up to 95% for PFOS and 94% for PFOA (Fig. 2). Mean and median sum of PFAS was 7.3 ng/ml (SD 4.6) and 6.2 ng/ml [IQR 4.4–9.2], respectively. Maximum detected concentration for the sum of PFAS was 35 ng/ml. PFOS isomers were the predominant contaminants in plasma, representing the highest contribution to total PFAS content, with a maximum detected concentration of 25 ng/ml. A graphical representation of all detected compounds is depicted in Fig. 3. Results were also embedded according to the NASEM (National Academies of Sciences, Engineering, and Medicine) guidelines in three reference values for total PFAS, <2 ng/ml, 2-20 ng/ml and ≥ 20 ng/ml. The sum of PFAS was below 2 ng/ml in 8.9% of cases, while 92.5% was in the range 2–20 ng/ml. Few samples (n. 6) displayed values above 20 ng/ml (1.4%) (Fig. 4).

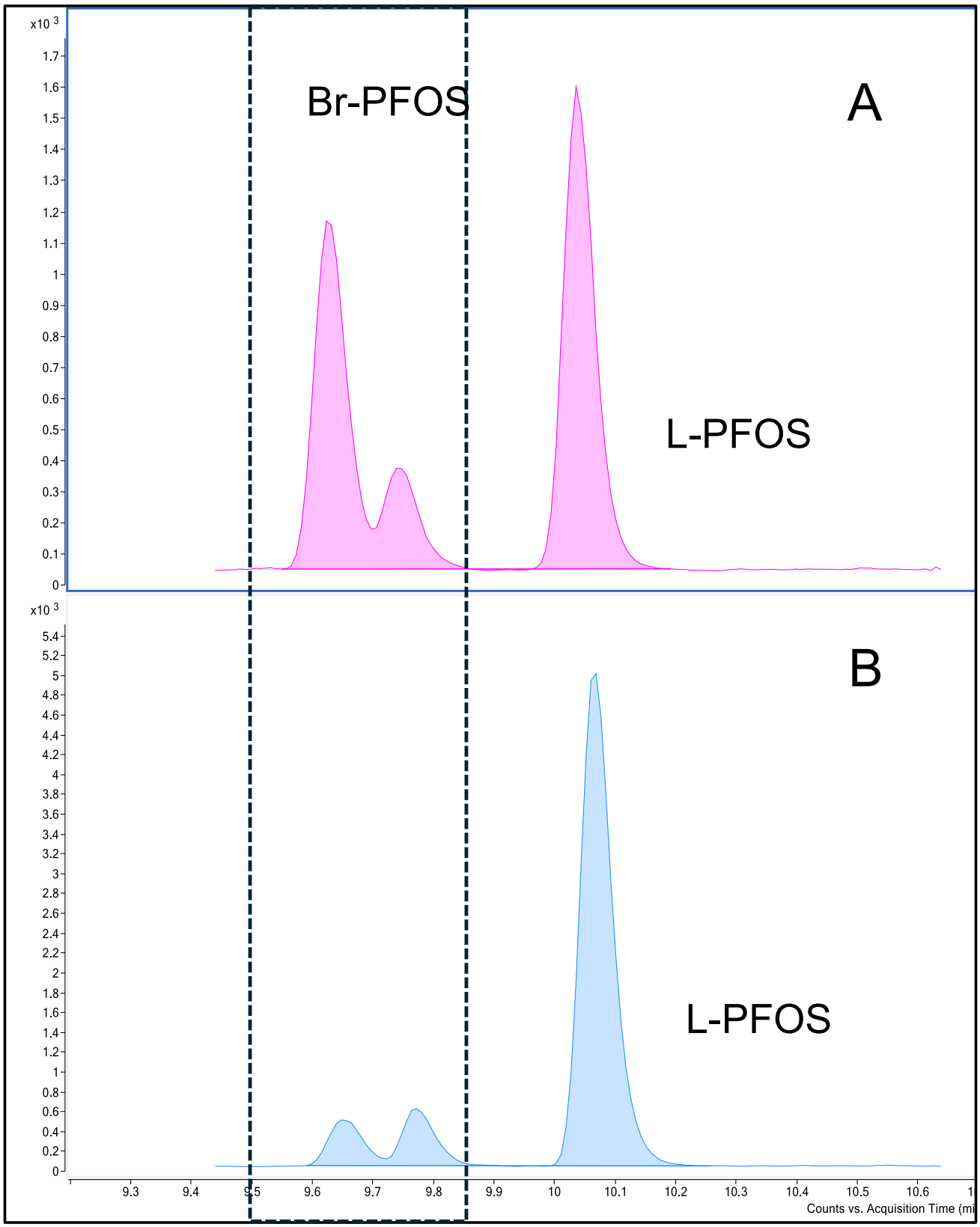

Fig. 1 EIC of m/z 498.9→80 quantifier transition (qualifier m/z 498.9→ 99 not shown) chromatogram of PFOS isomers in A) real sample at the concentration of 4.2 ng/ml and B) in reference material at the concentration of 10 ng/ml. L-PFOS: linear PFOS, br-PFOS: branched PFOS

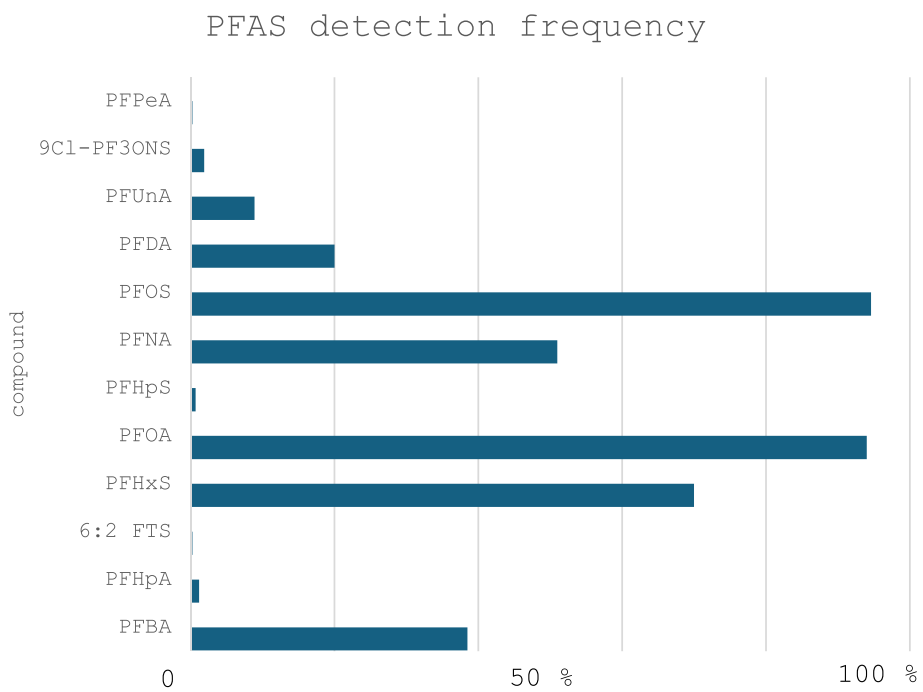

Fig. 2 Graphical representation of PFAS detection frequency in the 426-cohort analyzed

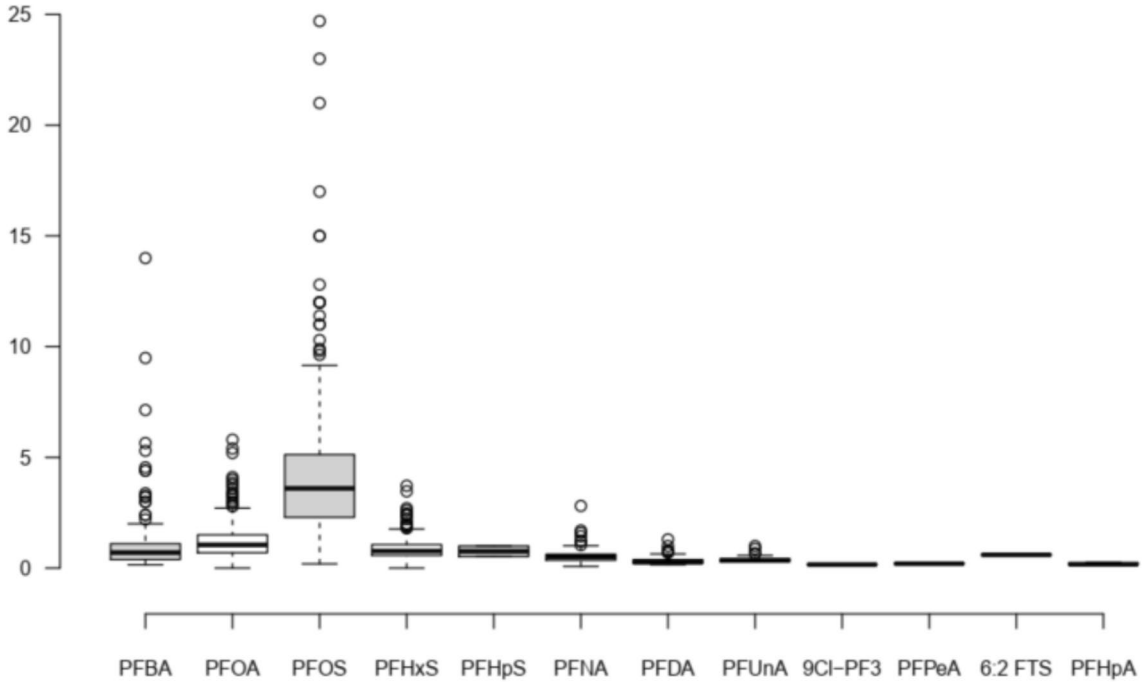

Fig. 3 Whiskers box plot distribution of PFAS among the analyzed population. Media and median values are represented by X and the bar, respectively. Y-axis: ng/ml

Discussion

Analytical methods for PFAS analysis in biological samples, such as blood or its derivatives, are among the most recent to be developed and are largely derived from methods originally optimized for complex non-biological matrices, where solid-phase extraction (SPE) was commonly used as the standard sample preparation approach. Classical WAX-SPE plasma/serum methods typically offer strong clean-up but require multiple steps, which increase analysis time and introduce more potential PFAS background sources. Originally PFAS plasma analysis had been proposed after sample purification by solid-phase extraction

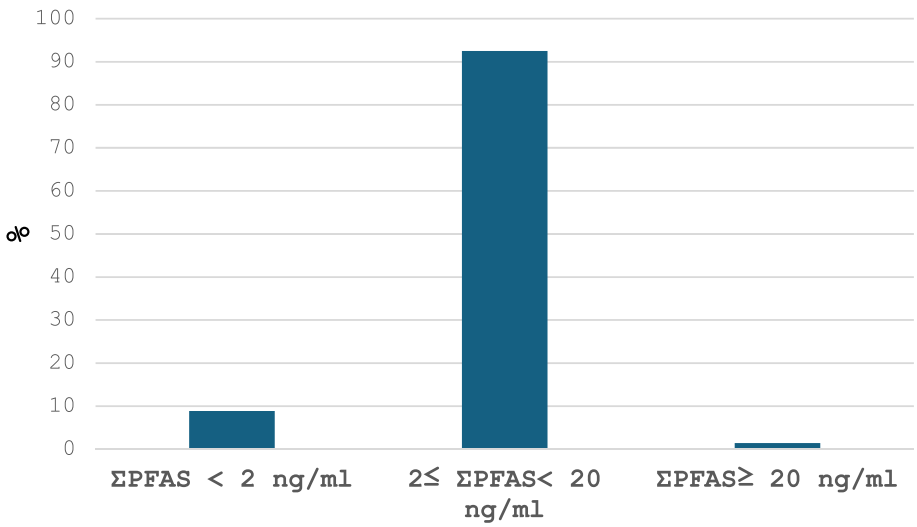

Fig. 4 NASEM classification of the retrieved PFAS concentrations

(SPE) (Kaiser et al. 2021), or a combination of protein precipitation and online-SPE (Papadopoulou et al. 2022), but faster protein-precipitation methods are becoming popular in literature, also in consideration of more sensitive instrumentation. The herein developed protein-precipitation LC–MS/MS method showed extraction and validation performances fully comparable with, and in some respects advantageous over some SPE-based PFAS plasma/serum methods (Kato et al., 2018), while supporting large-scale biomonitoring on 426 samples confirming long-standing instrument performance. Comparative research (Frigerio et al. 2022; Dui et al. 2024) showed that, for diverse PFAS panels, protein precipitation can outperform SPE in recovery and process efficiency. Our method provided recoveries between 86–102% with ion suppression below 30% for all analytes. Calibration curves were linear for several long-chain PFAS (e.g. PFUnA, PFOS, PFHxS, PFHpS, PFHpA, PFDoA, 9-Cl-PF3ONS) and quadratic for others, with correlation coefficients between 0.990 and 0.999. The LOQ (0.04–1.20 ng/ml) were comparable to or below those reported for validated high-throughput serum/plasma PFAS methods (Pitter et al. 2020; Salihovic et al. 2013), confirming that the absence of SPE does not impair sensitivity when isotope-dilution and optimized LC–MS/MS have been used. Accuracy (bias < 16%) and precision (RSD < 10% intra- and inter-day) suited the typical acceptance criteria for quantitative bioanalytical methods (often ≤ 15–20% for PFAS), indicating good trueness and repeatability over multiple days and concentration levels. The optimized chromatographic conditions included the ability to resolve linear and branched isomers of key compounds such as PFOS, PFOA, and PFHxS, a crucial capability for isomer-specific analysis. A different distribution of branched isomers between the reference standard and biological samples was observed, a finding that warrants further investigation. The application of this validated method to a cohort of 426 subjects yielded significant findings regarding PFAS exposure in this population. Twelve different PFAS compounds were detected, with a stark prevalence pattern. The most notable finding was the ubiquitous presence of PFOS and PFOA, which were detected in 95% of the samples. Total PFAS concentrations averaged 7.3 ± 4.6 ng/ml (median 6.2 ng/ml), with a maximum of 35 ng/ml. This burden was dominated by PFOS isomers, which alone reached up to 25 ng/ml. These NASEM guidelines categorize total PFAS levels into three reference values: less than 2 ng/ml (adverse effects not expected), 2–20 ng/ml (potential for adverse effects), and greater than or equal to 20 ng/mL (increased risk of adverse effects). The distribution of our cohort across these categories presented a critical public health finding. Only a small fraction, 8.9% of the cases, had total PFAS concentrations below 2 ng/ml. In contrast, a staggering 92.5% of the samples fell within the 2–20 ng/ml range, a level associated with a "potential for adverse effects". Fortunately, only a small number of samples (1.4%, n. 6) reached concentrations of 20 ng/ml or higher. A biomonitoring study carried out in the same geographical area on pregnant women before delivery showed mean PFOS, PFOA and PFHxS at lower concentrations, namely 0.64 ng/ml, 0.27 ng/ml and 0.11 ng/ml and no samples above 20 ng/ml for total PFAS; these findings confirm inter-individual variability in PFAS levels within populations from the same geographical region, highlighting the potential influence of lifestyle habits and occupational exposures on PFAS body burden (Sech et al., 2024). A comparative analysis of our results with data from the U.S. National Health and Nutrition Examination Survey (NHANES) 2017–2020 was performed to provide epidemiological consideration. In both studies, PFOS, PFOA and PFHxS were the most frequently detected compounds, revealing also similar pattern of concentrations, PFOS > PFOA > PFHxS. In the NHANES study, PFOS and PFOA linear and branched congeners were separately

quantified, but the calculated sum was in same order of magnitude of our study (PFOS 6.11 ng/ml vs 4.1 ng/ml and PFOA 1.68 ng/ml vs 1.2 ng/ml). PFNA was detected in our study in 58% of cases at lower average concentrations respect to other PFAS counterparts (PFNA 0.3 ng/ml). Exposure assessment in communities exposed to contaminated drinking water showed geometric mean serum concentration of PFOS, PFOA, and PFHxS respectively of 8.6 ng/ml (95% CI:8.3–8.9), 3.1 ng/ml (95% CI: 3.0–3.2), and 4.1 ng/ml (95% CI: 3.9–4.3), which were statistically higher than the general U.S population (Daly et al. 2018). Residents living in Italian contaminated sites (n. 6669) displayed PFOA mean concentration of 51.6 ng/ml, PFOS mean concentration of 4.1 ng/ml, PFHxS mean concentration of 3.6 ng/ml and PFNA mean concentration of 0.5 ng/ml (Canova et al. 2021). Another study referred to the Italian population sampled in a heavy contaminated area revealed plasma median PFOA, PFHxS and PFOS respectively of 44.4 ng/ml, 3.9 ng/ml and 3.9 ng/ml. These values were significantly higher for PFOA and PFHxS but comparable for PFOS (Pitter et al. 2020). The comparison between these two Italian populations suggests that the overall PFAS body burden in contaminated sites may have been predominantly influenced by specific compounds, particularly perfluorooctanoic acid (PFOA) and perfluorohexane sulfonate (PFHxS). This finding highlighted the likely pivotal role of these substances as major contributors to contamination patterns, reflecting both their historical use and environmental persistence. The biomonitoring results from this study have profound public health implications. The most prevalent compounds—PFOS, PFOA, and PFHxS—have been epidemiologically linked to various adverse health outcomes (Steenland et al. 2010). These associations include changes in cholesterol levels, altered liver enzyme function, and a lower antibody response to certain vaccines. Furthermore, specific compounds, like PFOA (IARC 2025), have been associated with an increased risk of kidney and testicular cancer. However, the interpretation of the study's findings must be approached with a clear understanding of its inherent limitations. The study's volunteer-based sample also introduces the potential for selection bias, as individuals who are concerned about their exposure may be more likely to participate, which could limit the generalizability of the findings to the broader population. Finally, while PFAS are persistent in the body, with a half-life (Batzella et al. 2024; Feng et al. 2025) of 2–7 years according to the molecule, a single plasma sample is merely a snapshot of the body burden at one moment in time and does not fully account for an individual's lifetime exposure history. Future research should transition from a cross-sectional to a longitudinal study design to more effectively capture the dynamic nature of exposure and its health impacts over time. This shift is essential for the biomonitoring approach, as it allows for the repeated measurement of biomarkers, providing a clearer understanding of accumulation, elimination, and long-term health effects. The future perspective of this study on Italian population will also include and consider lifestyle and occupational factors in relation to PFAS concentrations, in order to better define the potential contributors to PFAS exposure.

Conclusions

The present study proposed and applied a novel analytical method for the rapid and direct analysis of Per- and Polyfluoroalkyl substances (PFAS) in human plasma, demonstrating its utility for large-scale biomonitoring efforts on 426 subjects. This approach represented a significant advancement over traditional methodologies, which often involve complex and time-consuming SPE sample preparation steps. This simple sample handling not only accelerated the analytical workflow, but it also critically reduced the risk of background contamination due to the numerous steps and solvents used for SPE purification, a persistent challenge in trace-level PFAS analysis. The application of this method to a cohort of Italian population confirmed PFOS and PFOA detected in all samples, and 93% of the population falling into the category defined by NASEM guidelines with a potential for adverse health effects. Based on these findings, future research should transition from a cross-sectional to a longitudinal study design. Tracking PFAS levels in the same cohort over time would allow for an assessment of the temporal relationship between specific activities and changes in biomarker concentrations, thereby strengthening the evidence for a causal link.

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Data Availability Enquiries about data availability should be directed to the authors.

Declarations

Competing Interests The authors have not disclosed any competing interests.

Ethical Approval The study was approved by the Research Bioethical Board of University of Bologna (0041730 and 0007486), Italy.

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